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# Evolution of a novel 5-amino-acid insertion in the β3–β4 loop of HIV-1 reverse transcriptase

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**Abstract**

HIV-1 isolates harbouring an insertion in the β3–β4 loop of reverse transcriptase (RT) confer high-level resistance to nucleoside analogues. We have identified a novel 5-amino-acid insertion (KGSNR amino acids 66–70) in a patient on prolonged nucleoside combination therapy (didanosine and stavudine) and investigated which factors were responsible for its outgrowth. Remarkably, only small fold increases in drug resistance to nucleoside analogues were observed compared to wild type. The insertion variant displayed a reduced replicative capacity in the absence of inhibitor, but had a slight replicative advantage in the presence of zidovudine, didanosine or stavudine, resulting in the selection and persistence of this insertion *in vivo*. Mathematical analyses of longitudinal samples indicated a 2% *in vivo* fitness advantage for the insertion variant compared to the initial viral population. The novel RT insertion variant conferring low levels of resistance was able to evolve towards a high-level resistant replication-competent variant.



*Keywords:* HIV-1; Reverse transcriptase; Resistance; Insertion; Replication capacity

## Introduction

The human immunodeficiency virus type 1 (HIV-1) enzyme reverse transcriptase (RT) is an important target for antiretroviral treatment, due to its unique and essential role in the virus life cycle (Arts and Wainberg, 1996). Nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs) compete with the natural dNTPs for incorporation in the growing proviral DNA chain (Boyer et al., 2001). Once incorporated, further DNA synthesis is blocked, because NRTIs lack the 3′-hydroxyl group essential for chain elongation. Unfortunately HIV is able to develop resistance against all NRTIs. Crystallography studies have shown that the amino acid substitutions causing resistance are located around the nucleotide-binding site. They confer resistance against NRTIs via two mechanisms: discrimination between the nucleotide analogue and its natural counterpart (dNTP) or enhanced excision of the incorporated nucleotide analogue (Huang et al., 1998; Selmi et al., 2003). Continued treatment with NRTIs can lead to the development of cross-resistance to the complete drug class. Three pathways have been described that confer class resistance. Accumulation of mutations associated with NRTI cross-resistance, such as M41L, D67N, K70R, L210W, T215Y/F and K219Q/E, is the most prevalent pathway (Matsumi et al., 2003). Another pathway is referred to as the Q151M pathway, in which the Q151M substitution is selected, in combination with amino acid changes A62V, V75I, F77L and F116Y (Deval et al., 2002; Kaushik et al., 2000). The third pathway involves the selection of an insertion between codons 68 and 69 or 69 and 70 and has an estimated prevalence of less than 3% in therapy-failing

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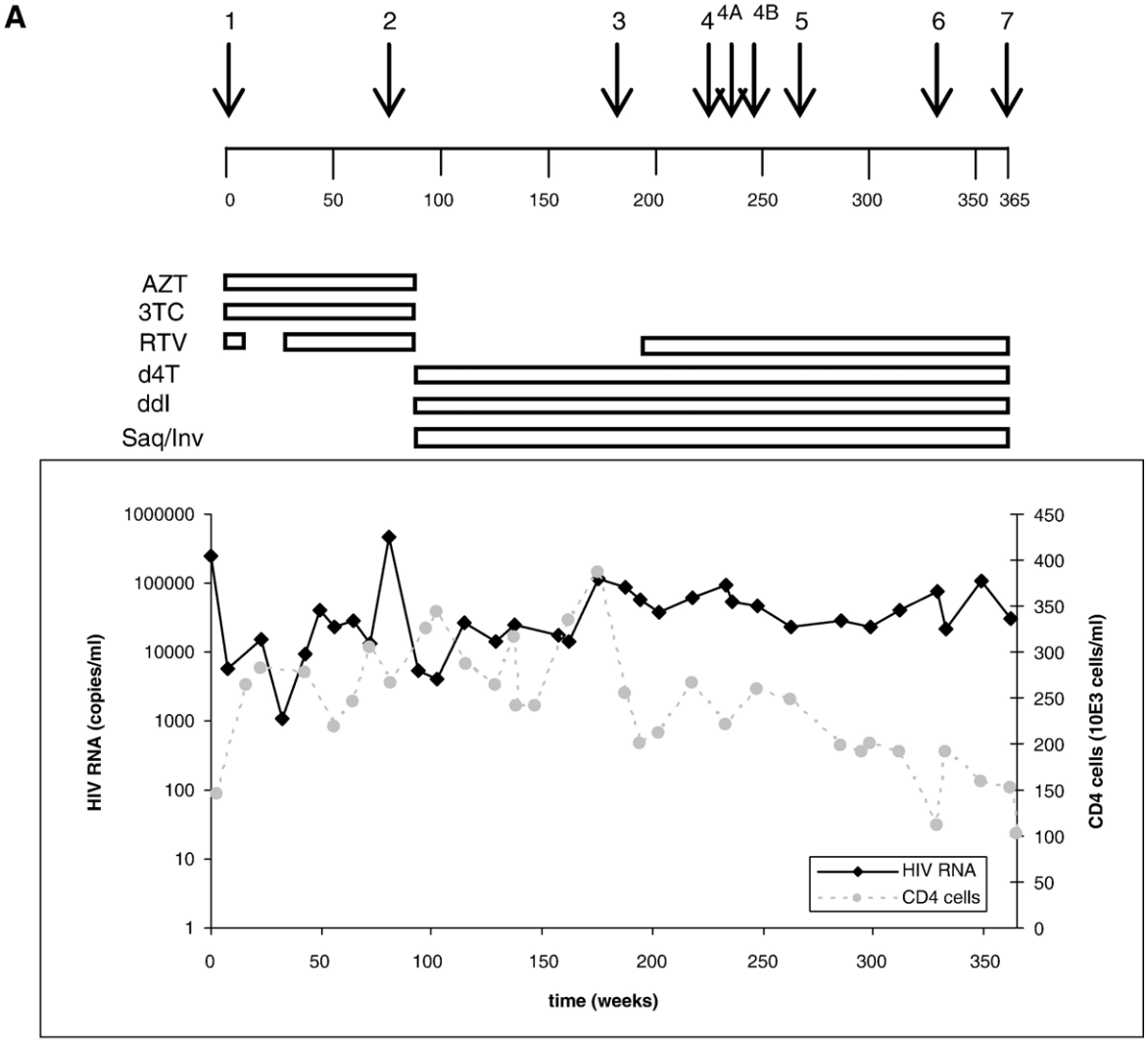
patients (Tamalet et al., 2000; Larder et al., 1999; Winters et al., 1998; Ross et al., 1999; Sugiura et al., 1999). These insertions are mostly dipeptides, mainly SS, SG or SA, although single amino acid as well as three to eleven amino acid insertions have also been described (Sato et al., 2001; Lobato et al., 2002; van der Hoek et al., 2005). Such insertions




| amino acid in RT | 20 | 27 | 28 | 32 | 33 | 35 | 39 | 60 | 64 | 66 | 67 | 68 | 69 | 70 | insertion | 83 | 101 | 118 | 122 | 139 | 166 | 175 | 177 | 184 | 196 | 211 | 214 | 215 | 219 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| wild type (consensus B) | K | T | E | K | A | V | T | V | K | K | D | S | T | K | | R | K | V | K | T | K | N | D | M | G | R | F | T | K |
| sample 1 | K/R | - | - | - | - | - | - | I | - | - | - | - | N | R | - | K | - | I | - | L | R | H | - | - | - | K | L | - | - |
| sample 2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | K | - | I | - | S | - | - | - | V | - | K | L | - | - |
| sample 3 | R | - | - | - | - | I/V | - | I | - | - | G | - | N | R | - | K | Q | I | E | L | R | - | - | - | - | K | L | - | Q |
| sample 4 | R | - | - | - | A/G | I/V | - | I | - | - | G | - | N | R | - | K | Q | I | E | L | R | - | - | - | - | K | L | - | Q |
| sample 4 clone 18 (S4C18) | R | - | - | - | G | I | - | I | - | - | G | - | N | R | - | K | Q | I | E | L | R | - | - | - | - | K | L | - | Q |
| sample 5 | R | - | - | - | - | I | - | I | - | - | G | - | N | R | KGSNR | K | Q | I | E | L | R | - | - | - | - | K | L | I | Q |
| sample 5 clone 22 (S5C22) | R | A | - | - | - | I | - | I | - | - | G | - | N | R | KGSNR | K | Q | I | E | L | R | - | - | - | - | K | L | I | Q |
| sample 6 | R | - | - | E | - | R/T | A/T | I | K/R | - | G | D | N | R | KGSNR | K | Q | I | E | L | K/R | - | - | - | - | K | L | F/T | Q |
| sample 6 clone 3 (S6C3) | R | - | - | - | - | - | A | I | - | - | G | - | N | R | KGSNR | K | Q | I | E | L | R | - | - | - | E | K | L | - | Q |
| sample 6 clone 4 (S6C4) | R | - | - | E | - | R | - | I | - | - | G | D | N | R | KGSNR | K | Q | I | E | L | - | - | E | - | - | K | L | F | Q |
| sample 6 clone 10 (S6C10) | R | - | G | - | - | R | - | I | - | - | G | D | N | R | KGSNR | K | Q | I | E | L | R | - | - | - | - | K | L | - | Q |
| sample 7 | R | - | - | E | - | R/T | A/T | I | K/R | - | G | D | N | R | KGSNR | K/R | Q | I | E | L | - | - | - | - | - | K/R | L | F/T | Q |

B

| amino acid in RT | 64 | 65 | 66 | 67 | 68 | 69 | 70 | - | - | - | - | - | 71 | 72 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| consensus B | K | K | K | D | S | T | K | - | - | - | - | - | W | R |
| wild type | AAG | AAA | AAA | GAC | AGT | ACT | AAA | - | - | - | - | - | TGG | AGA |
| sample 4 | K | K | K | **G** | S | **N** | **R** | - | - | - | - | - | W | R |
| | AAG | AAA | AAA | G**G**C | AGT | A**A**T | A**G**A | - | - | - | - | - | TGG | AGA |
| sample 5 | K | K | K | **G** | S | **N** | **R** | K | **G** | S | **N** | **R** | W | R |
| | AAG | AAA | AAA | G**G**C | AGT | A**A**T | A**G**A | AAA | G**G**C | AGT | A**A**T | A**G**A | TGG | AGA |

Fig. 1. (A) Viral load, $CD4^+$ count, treatment of patient M17001 and genotypic analyses. HIV-1 RNA copies and total CD4 counts were measured at several time points. The drug therapy is shown on top. At several time points during the treatment of patient M17001, as indicated by the black arrows, the plasma viral population was sequenced. For samples 4, 4A, 4B, 5 and 6 viral clones were generated and sequenced as well. (B) Nucleotide sequence of the 5-amino-acid insertion in RT. The nucleotide sequences and corresponding amino acids 64 to 72 of HIV-1 RT are shown for sample 4 and sample 5. The insertion is an exact duplication of the previous 15 nucleotides. AZT, zidovudine; 3TC, lamivudine; RTV, Ritonavir; d4T, stavudine; ddI, didanosine; SAQ, Saquinavir.

localize in the β3–β4 loop of the fingers subdomain of HIV-1 RT, which plays a key role in the functioning of the polymerase by positioning the template and incoming nucleotide (Fisher et al., 2003). Genotypic analysis indicates that these insertions are usually duplications of prior codons, supporting the general opinion that generation of insertions is explained by slippage of RT at a pausing site (Harrison et al., 1998). The selection of insertions usually occurs during treatment with at least one thymidine analogue (d4T and/or AZT). They generally appear in the background of specific thymidine associated mutations (TAMs), such as the M41L, K70R, L210W and T215Y/F changes and are highly associated with A62V, D67E/G and T69S substitutions (Ross et al., 1999; Lukashov et al., 2001; Moyle et al., 2000). When accompanied by (a part of) these mutations the insertion complex invariably causes high-level resistance to all approved nucleoside analogues. The precise mechanism through which these insertions cause resistance is not clear. Although the insertion is able to increase the discrimination of a nucleotide analogue, the major mechanism of resistance for the insertion complex appears to be the increased excision of NRTIs from their terminated primer (Meyer et al., 2003; Boyer et al., 2002; Mas et al., 2000).

We have identified an HIV-1 variant harbouring a stable novel 5-amino-acid insertion in the fingers domain of RT in a patient failing on nucleoside combination therapy. In this study we have analyzed which factors drive the selection of this novel insertion variant.

## Results

### *Genotypic analysis*

Longitudinal plasma samples of patient M17001 were genotypically analyzed as shown in Fig. 1. At 263 weeks after start of highly active antiretroviral therapy (HAART) the viral population sequence (sample 5) revealed a very large, unique insertion of 15 nucleotides around codon 66 in RT. These 15 nucleotides encoded for amino acids KGSNR; an exact duplication of amino acids 66 to 70 (Fig. 1B). This 5-amino-acid insertion was selected in the background of the D67G, K70R, V118I, K219Q and the unusual T69N and T215I amino acid changes. Several other mutations that are not associated with an insertion in the fingers domain of RT were present in the viral background as well (Fig. 1). Selection of the insertion in RT did not coincide with substitutions in the C-terminal part of RT and/or protease (data not shown).

During sustained treatment with stavudine (d4T) and didanosine (ddI) the T215I substitution evolved towards a mixture of phenylalanine and threonine, whereas the 5-amino-acid insertion remained stably present in the viral population (Fig. 1). In this study we wanted to investigate which factor(s) is (are) driving the selection of this unique insertion, and therefore we determined its effect on replication capacity and drug susceptibility.

### *Replication capacity of the novel insertion variant*

To investigate if the selection of the 5-amino-acid insertion variant is caused by an increase in replication capacity a recombinant virus clone was generated, which was representative of the viral population (sample 5 clone 22; S5C22 (insert)). For comparison a representative recombinant virus clone of sample 4 was generated (sample 4 clone 18; S4C18, Fig. 1).

Replication competition experiments demonstrated a negative effect on replication capacity, since S4C18 completely replaced the insertion-containing virus S5C22 (insert) clone (Fig. 2). Moreover, *in vitro* evolution experiments demonstrated that the 5-amino-acid insertion was deleted after 65 days when the virus clone S5C22 (insert) was cultured in PBMC in the absence of RT inhibitors, while all other amino acid changes remained stably present (data not shown). Thus, in the absence of drug pressure the replication capacity of the insertion variant is decreased compared to its predecessor,

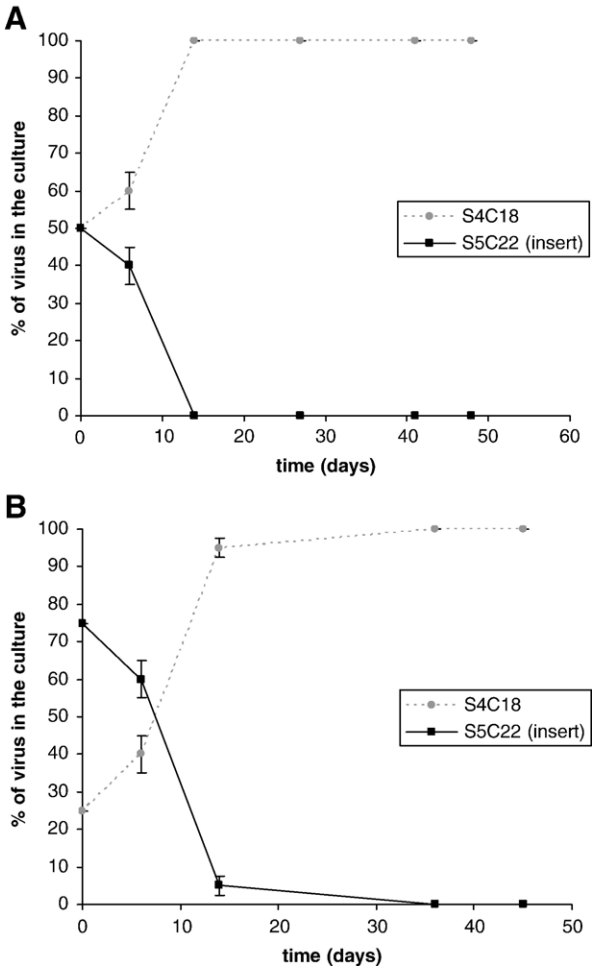



Fig. 2. Competition experiments between S4C18 and S5C22 (insert). S4C18 (sample 4 clone, without insertion) was competed with S5C22 (sample 5 clone, with insertion) in PBMC for 5 passages. At several time points the percentage of both viruses in the culture was determined by sequencing as described in the Materials and methods section. Shown are two independent experiments that were started at a 50:50 (A) or 75:25 ratio (B) based on TCID50. The variability is indicated by error bars representing the standard error of the mean (SEM).

indicating that another factor is driving the selection of this novel insertion.

*Drug susceptibility analysis*

To determine the impact of this novel insert variant on drug susceptibility the recombinant virus clones S5C22 (insert) and S4C18 were phenotypically analyzed. Results of at least duplicate experiments are shown in Table 1B. To our surprise, no high-level resistance for S5C22 (insert) could be determined for the three NRTIs tested. The moderate reductions in susceptibility to AZT, d4T and ddI were respectively 9-, 3-, and 2-fold.

Our results were confirmed by the phenotypic assays from Monogram Biosciences (formerly ViroLogic; PhenoSense assay) and VIRalliance (PhenoScript assay). In these assays no high-level resistance could be observed for S5C22 (insert) as well (data not shown).

A small increase in resistance was observed for S5C22 (insert) compared to S4C18 for AZT in PBMC (9-fold compared to 3-fold), whereas no difference could be measured for d4T and ddI. However, small increases in drug resistance in PBMC were measured when we determined the viral replication capacity under drug pressure (Fig. 3). The level of virus replication in the presence of serial dilutions of either d4T, ddI or AZT was assessed and for all drugs tested, an increased replication in the presence of inhibitor was observed for S5C22 (insert) as compared to S4C18. This means that at the higher drug concentrations the insertion variant (S5C22 (insert)) confers a higher replication capacity than the non-insertion variant (S4C18), indicating a small replicative advantage for the insertion-variant under d4T, ddI or AZT pressure in PBMC.

*Modeling of the 5-amino-acid insertion in RT and its putative mechanism of resistance*

To obtain possible insights into the three-dimensional structure of this large insertion and its corresponding mechanism of resistance, a 3D model was generated (Fig. 4). Because of the surface location of R70, the 5-residue insertion can be accommodated relatively easily in the RT structure. The minimized model gave indications of binding of the duplicated R70 to the triphosphate moiety of the substrate (dTTP) and by extrapolation, to the inhibitor (AZT-TP) and also to the ATP used for excision of the blocked primer, in the binding mode modeled in previous reports (Boyer et al., 2001; Chamberlain et al., 2002). To determine if the second positively charged arginine is indeed responsible for the increase in resistance, a

Table 1
Drug resistance analyses for several viral HIV-1 clones derived from patient M17001

(A)

| Consensus B | 66 K | 67 D | 68 S | 69 T | 70 K | | | | | 118 V | 215 T | 219 K |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S4C18 | | G | | N | R | | | | | I | | Q |
| S5C22 | | G | | N | R | K | G | S | N | R | I | I | Q |
| S5C22-R70A | | G | | N | R | K | G | S | N | A | I | I | Q |
| S6C3 | | G | | N | R | K | G | S | N | R | I | | Q |
| S6C4 | | G | D | N | R | K | G | S | N | R | I | F | Q |
| S6C10 | | G | D | N | R | K | G | S | N | R | I | | Q |

(B)

| PBMC | AZT | | ddI | | d4T | |
|---|---|---|---|---|---|---|
| | IC50 (nM) (mean±SEM) | Fold increase in IC50 | IC50 (nM) (mean±SEM) | Fold increase in IC50 | IC50 (nM) (mean±SEM) | Fold increase in IC50 |
| HXB2 (wild type) | 23±9 | | 1894±451 | | 223±73 | |
| S4C18 | 70±24 | 3 | 2729±795 | 1 | 739±192 | 3 |
| S5C22 | 209±100 | 9 | 3347±487 | 2 | 719±99 | 3 |
| S6C3 | 288±230 | 13 | 5297±2285 | 3 | 702±390 | 3 |
| S6C4 | 2592±1469 | 113 | 4296±591 | 2 | 1783±1351 | 8 |
| S6C10 | 2030±409 | 88 | 3970±783 | 2 | 953±112 | 4 |

(C)

| MT2 cells | AZT | |
|---|---|---|
| | IC50 (nM) (mean±SEM) | Fold increase in IC50 |
| S5C22 | 2321±807 | |
| S5C22-R70A | 1179±340 | 0.5 |

Five representative viral clones from patient M17001 were analyzed for their susceptibility for several reverse transcriptase inhibitors. Sample 4 clone 18 (S4C18) represents a viral clone without the insertion and sample 5 clone 22 (S5C22) contains the novel 5-aa insertion. Sample 6 clones correspond to the evolved virus variant in the patient. To determine the mechanism of resistance the second arginine at position 70 (of the insert) was replaced by a neutral alanine, resulting in virus clone S5C22-R70A.

Shown are relevant amino acid changes (A) the mean $IC_{50}$ values compared to a wild type reference virus (HXB2) in PBMC (B) or compared to S5C22 in MT2 cells (C). AZT; zidovudine, d4T; stavudine, ddI; didanosine.

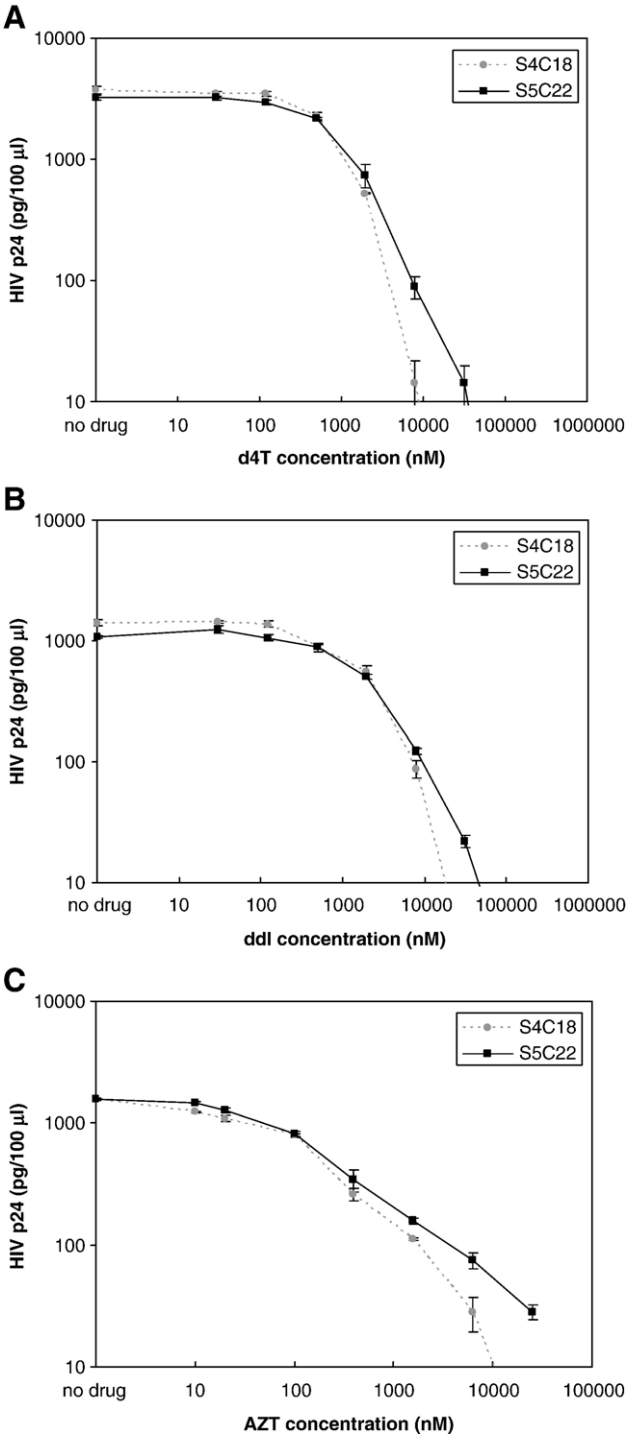



Fig. 3. Replication capacity under drug pressure. The replication capacity of S4C18 and S5C22 was determined under a serial dilution of either d4T (A), ddI (B) or AZT(C). The amount of viral production in PBMC after 7 days, measured by P24 ELISA was plotted in a graph. Representative experiments are shown. In each experiment the virus production was measured in triplicate; the variability is indicated by error bars representing the standard error of the mean (SEM).

site-directed mutant was generated in which the arginine was replaced by an uncharged alanine (A) (S5C22-R70A; Table 1A). It was demonstrated that the replacement of the second arginine by an alanine indeed resulted in a two-fold decrease in $IC_{50}$ for AZT (Table 1C).

*In vivo fitness and evolution of the insertion variant*

To determine the fitness advantage of this novel insertion *in vivo*, viral clones were generated as described previously and genotypically analyzed to determine the percentage of virus in the population that contains the insertion. In sample 4A only 1 out of the 20 clones (5%) contained the insertion, while sample 4B revealed that 20% of the viral population harboured the insertion (4/20 clones). These clonal frequencies in combination with the viral load over time were used to estimate that the *in vivo* fitness advantage of the insertion mutant was 2.1% (±0.6%).

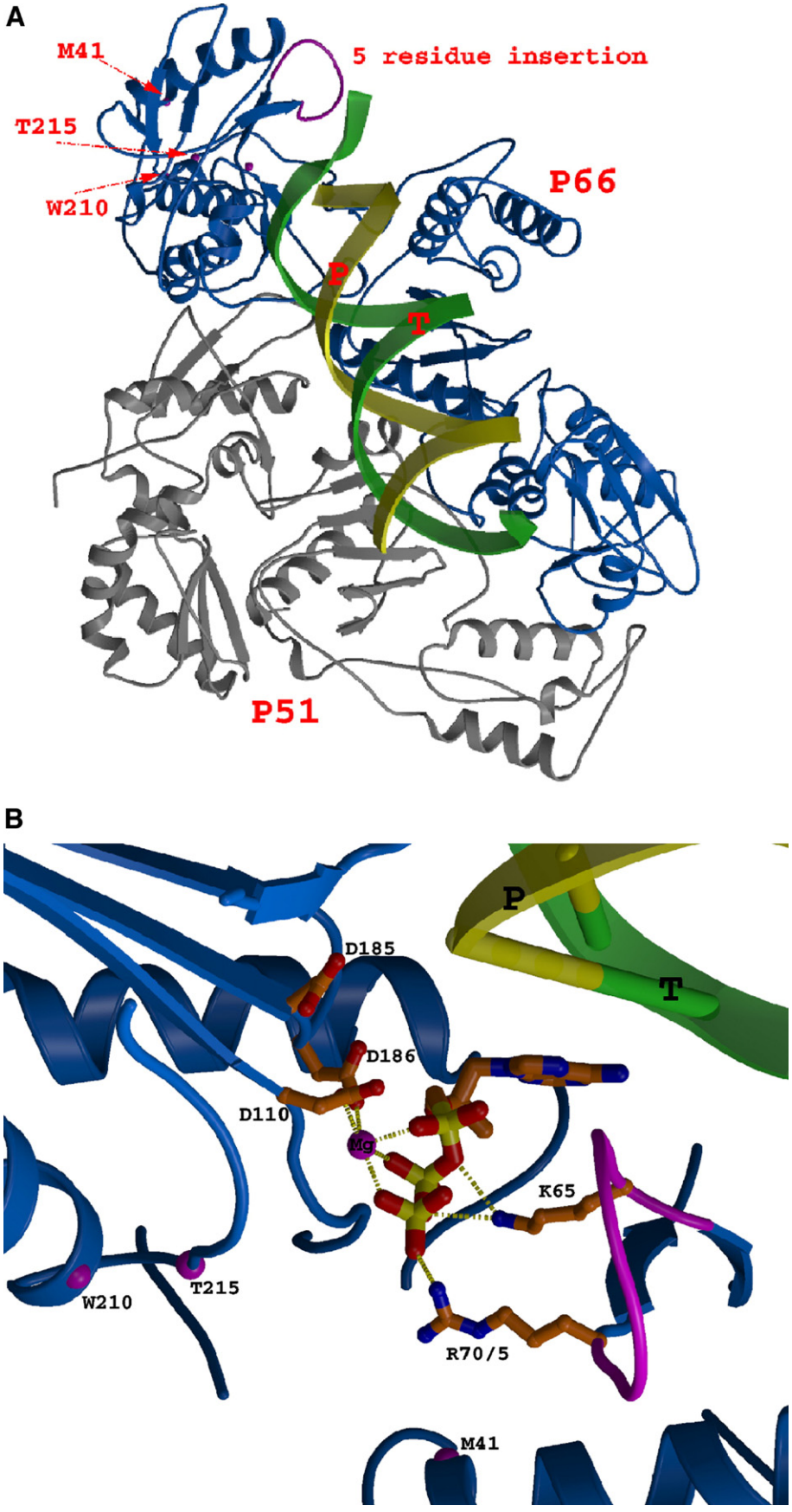
We have shown that the selection of this insertion is driven by a small increase in drug resistance. However, since the effect on drug resistance was rather small we investigated if the virus was able to evolve towards a better replicating and/or more resistant variant under continued drug pressure in the patient. Longitudinal plasma samples from patient M17001, who remained on the same therapy, were sequenced (Fig. 1). We demonstrated that the KGSNR insertion remains stably present in the patient. However, continuous viral evolution was observed, as indicated by the acquisition of a serine-to-aspartate substitution at position 68 (S68D). This double transition amino acid change was stably selected whereas the isoleucine (I) at codon 215 was replaced by a mixture of phenylalanine (F) and the wild type threonine (T). Recombinant virus clones representing these different viral variants were generated from sample 6 (S6C3, S6C4 and S6C10) and their susceptibility to AZT, d4T and ddI was determined in PBMC (Table 1). As shown in Table 1B the evolved insertion variants conferred high-level resistance against both AZT and d4T. Moreover, replication competition experiments using S6C4 and S5C22 demonstrated a replicative advantage for S6C4, which indicates that the replication capacity is increased as well (Fig. 5). Analysis of the reverse transcriptase activity of the virus clone before selection of the insertion (S4C18), the initial insertion variant (S5C22) and the evolved insertion variant (S6C4) revealed the same trend as described for replication capacity (Fig. 6).

## Discussion

In this study we investigated the evolution of a novel insertion in RT (KGSNR; duplication of amino acids 66–70) that was selected in a patient on prolonged non-suppressive nucleoside combination therapy in the background of multiple RT mutations (D67G, T69N, K70R, V118I, T215I and K219Q). We studied longitudinal samples of this patient and investigated recombinant virus clones for their effect on replication and drug susceptibility.

The insertion variant displayed a reduced replicative capacity in the absence of inhibitor, but had a slight replicative advantage in the presence of didanosine or stavudine, resulting in the selection and persistence of this insertion *in vivo*. The insertion variant was able to evolve towards a replication-competent, high-level resistant variant under continued drug pressure.

Although there is still debate about the effect of insertions on processivity, the general opinion holds that insertions in the


A
M41
5 residue insertion
T215
P66
W210
P
T
P51
B
P
T
D185
D186
D110
Mg
K65
W210
T215
R70/5
M41

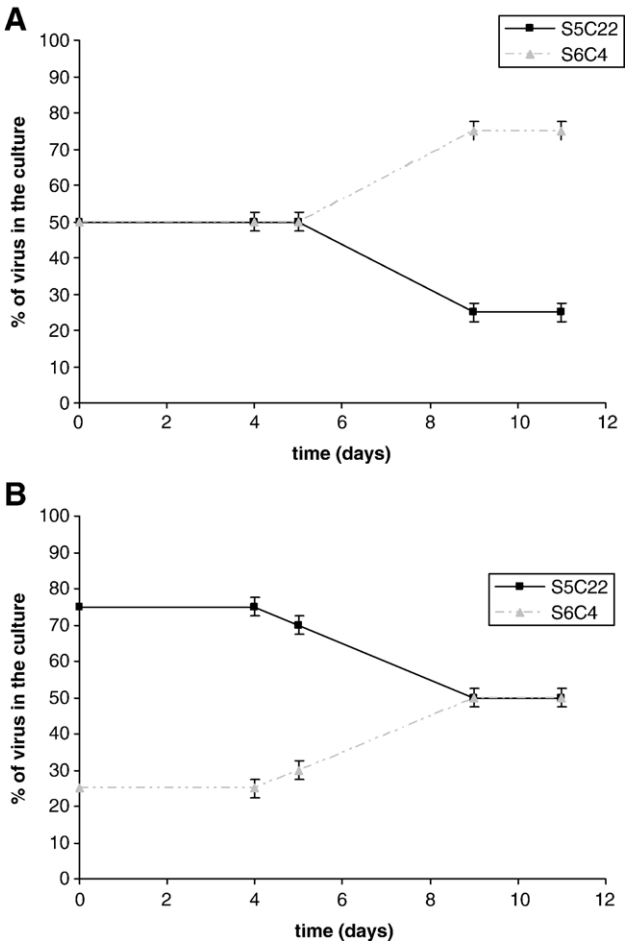



Fig. 5. Competition experiments between S5C22 and S6C4. S5C22 (sample 5 clone, with insertion) was competed with S6C4 (sample 6 clone 4, *in vivo* evolved insertion variant) in Sup-T1 cells for two passages. At several time points the ratio of the two viruses in the culture was determined by sequencing. Results are two independent experiments that started with either a 50:50 ratio (A) or a 75:25 ratio (B) based on TCID50. The variability is indicated by error bars representing the standard error of the mean (SEM).

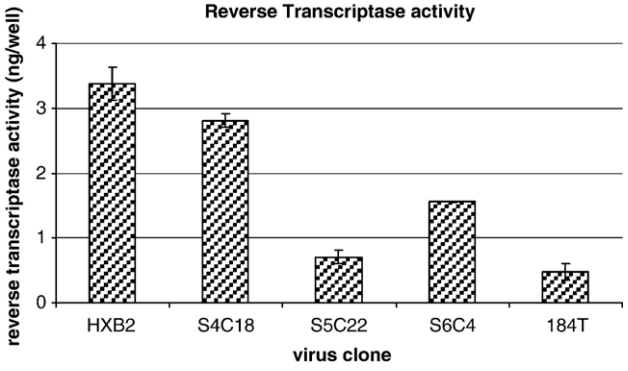



Fig. 6. Reverse transcriptase activity. The reverse transcriptase activity was determined using a colorimetric RT activity kit as described in the Materials and methods section. S4C18 (sample 4 clone, without insertion), S5C22 (sample 5 clone, with insertion) and S6C4 (sample 6 clone, evolved insertion variant) were analyzed. HXB2 (wild type reference strain) and 184T (reference strain with single M184T change) were used as a control for a respectively wild type or severely decreased reverse transcriptase activity. As input 20 ng p24 was used for each virus clone. The variability is indicated by error bars representing the standard error of the mean (SEM).

β3–β4 loop of RT result in a lower replication capacity and a defect in the efficiency of nucleotide incorporation or processivity (Boyer et al., 1999, 2002; Sato et al., 2001; Mas et al., 2000; Kew et al., 1998; Lukashov et al., 2001). In this study, we described a novel 5-amino-acid (KGSNR) insertion that resulted in a reduction in viral replication capacity and RT activity, which is in agreement with previously described insertions.

A striking difference however was the low level of resistance that was observed for this new variant. All other patient-derived virus variants harbouring an insertion in the β3–β4 loop that have been described demonstrated high-level resistance to all nucleoside analogues (Eggink et al., 2007; Boyer et al., 1999, 2002; Sato et al., 2001; Mas et al., 2000; Kew et al., 1998; Lukashov et al., 2001). Only *in vitro* engineered (serine) insertions without additional (resistance) mutations in reverse transcriptase were not able to decrease nucleoside analogue susceptibility (Quinones-Mateu et al., 2002). Our results imply that *in vivo* selected insertions in the β3–β4 loop of RT are not high-level resistant by definition.

Modeling studies and resistance assays revealed that the additional positive charge by the (second) arginine was responsible for a 2-fold increase in AZT resistance. The effects observed are, however, rather small and thus deciding which triphosphate binding may be affected is not straightforward, particularly given the flexibility of this region. Nevertheless, the second arginine at position 70 does at least appear partly responsible for the observed resistance against AZT.

We may be able to explain the low resistance profile of our insertion variant based on the absence of accompanying resistance mutations. Namely, considering all published sequences of *in vivo* selected RT insertion variants, we noticed that our unusual KGSNR insert variant did not contain the "usual" insertion-accompanying substitutions. Most insertion variants harbour a T69S and T215Y change and are highly associated with the M41L, L210W, A62V and D67E/G

Fig. 4. Modeling of the 5-amino-acid insertion in reverse transcriptase. (A) Cartoon representation of the overall architecture of HIV-1 RT with bound template: primer DNA generated from 1RTD pdb coordinates. The p51 subunit is colored silver, the p66 subunit is blue, while the template:primer DNA strands are shown in green and yellow respectively. The 5-amino-acid insertion (KGSNR) in the fingers domain of p66 is illustrated in magenta. The location of amino acids commonly associated with resistance to thymidine analogues (M41, T215 and W210) is shown as magenta spheres. (B) Cartoon illustration of the proposed interaction of residues within the 5-amino-acid insertion in the fingers domain of p66 generated from modeling studies. The main chains are shown in schematic form colored in blue; the 5-amino-acid insertion is highlighted in magenta; the template:primer strands in green and yellow respectively. The catalytic triad (D110, D185 and D186) along with residues K65 and R70 of the insertion and the dTTP substrate are illustrated as ball and stick representations. A magnesium ion and the location of residues T215 and W210 are shown in magenta. Ionic and/or hydrogen-bonding interactions including that proposed for the duplicated R70 with the substrate triphosphate are shown as broken yellow lines.

mutations (Boyer et al., 1999, 2002; Sato et al., 2001; Mas et al., 2000; Kew et al., 1998; Lukashov et al., 2001). Remarkably, of all these substitutions only the latter one is present in our insertion variant. Unfortunately, the exact function of this specific amino acid in insertion-containing variants is unknown, despite the fact that almost all described RT insertions are accompanied by a substitution at this particular codon. With respect to the M41L and T215Y, which are lacking in our insertion-variant, data have shown that they play a critical role by increasing the rate of ATP-dependent primer unblocking and thereby resistance (Boyer et al., 2002). Furthermore, also selection of the flanking T69S (in our variant: T69N) is suggested to increase polymerase activity and processivity (Boyer et al., 1999). Considering that the novel insertion variant in this study did not harbour any of these changes, the obtained low resistance profile seems plausible.

Interestingly, when our variant evolved towards a high-level resistant virus, still none of these known substitutions (e.g., M41L, T215Y, L210W, T69S) was selected. Apparently, selection of other "unknown" mutations in the viral reverse transcriptase enzyme can also increase the resistance profile of an insertion variant. This was also demonstrated by Bulgheroni et al. who have described two unusual patient-derived RT insertion variants, that were lacking most classical mutations as well, but conferred high-level resistance (Bulgheroni et al., 2004). We therefore hypothesize that the specific genetic background and the nature of the insertion itself will impact the evolutionary pathway to improve fitness. When our variant evolved towards a high level of resistance two amino acid changes were consistently observed. A mixture of 215F/T was selected, but a comparison of two clonal isolates with and without this change, demonstrated that that this mutation did not seem to play a critical role in resistance. The acquisition of the serine-to-aspartate substitution at codon 68 did increase the resistance to AZT towards very high levels. This S68D change is the result of two transitions and is hardly ever observed before in (un)treated HIV-1 subtype B isolates (hivdb.stanford.edu). To our knowledge, this amino acid change has been described once in a Korean HIV-1 subtype B isolate, which interestingly harboured the T69N as well (Cho et al., 2002).

Several studies have demonstrated that the initially impaired replication capacity of RT insertion variant can be (partly) compensated. For instance, the sequence of the insertion has been shown to be responsible for subtle differences in the rates of excision and replication capacity. An SS insertion has been described that evolved towards an SG insertion that revealed a higher excision rate and an advantage in *in vitro* polymerase assays (Boyer et al., 1999, 2002; Quinones-Mateu et al., 2002). Furthermore, the background of the insertion virus has been shown to be important as well. Previous studies demonstrated that recombinant viruses containing a clinical RT insertion variant revealed a higher replication capacity when compared to a site-directed mutant containing the same insertion in a wild type backbone(Prado et al., 2004; Quinones-Mateu et al., 2002, indicating that the presence of particular amino acids (at unknown positions) may influence the replication capacity of an insert in RT. Apparently, HIV-1 is capable of (partly) compensating for the insertion by the selection of mutations, which not necessarily have to be the "known" resistance mutations. Also in our study, we have demonstrated that low level resistant insertion variant with a low replication capacity is able to evolve towards a high-level resistant replication-competent variant by selecting novel mutations, such as S68D, in reverse transcriptase.

It could be questioned that the continuous pressure of protease inhibitors (also) played a role in the selection of this novel insertion. However, clonal sequencing has demonstrated that the insertion is not co-selected with a protease inhibitor resistance mutation, but has a selective fitness advantage on its own. Also, although we have sequenced the complete reverse transcriptase and protease genes of sequential plasma samples, we cannot exclude the influence of putative mutations in other genes. But, we believe that it is unlikely that the insertion is co-selected with other mutations because of its dramatic influence on replication capacity. Furthermore, mutations in reverse transcriptase can also be selected when they confer cytotoxic T lymphocyte (CTL) escape (Goulder et al., 1997). Again, this appears unlikely since the insertion does not disrupt a known CTL epitope in RT according to the Los Alamos database (http://hiv-web.lanl.gov).

In conclusion, we have investigated the factors driving the selection of a novel KGSNR insertion at position 66 in RT. It was demonstrated that the insertion resulted in a decrease in viral replication capacity, but showed a small fitness advantage at relatively high drug concentrations (d4T, ddI and AZT). Under sustained drug pressure the virus was able to evolve towards a high-level resistant variant with a higher replication capacity.

## Materials and methods

### *Cells*

Peripheral blood mononuclear cells (PBMC) were isolated from blood of five HIV-negative human donors (Sanquin Blood bank, Utrecht, The Netherlands) by means of Ficoll-Paque (Amersham Biosiences, Roosendaal, The Netherlands) gradient centrifugation. Aliquots of the pooled cells were frozen at −130 °C in RPMI 1640 medium with L-glutamine (Cambrex, Verviers, Belgium), supplemented with 20% heat-inactivated fetal calf serum (FCS, Invitrogen), 10% dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and 10 μg/ml gentamicin (Invitrogen). Three to 4 days before use, the cells were cultured in RPMI 1640 medium, supplemented with L-glutamine, 10% FCS and 10 μg/ml gentamicin and stimulated with 2 μg/ml phytohemagglutinin (PHA, Sigma, St. Louis, USA).

MT-2 cells and Sup-T1 cells were cultured in RPMI 1640 medium, supplemented with L-glutamine, 10% FCS and 10 μg/ml gentamicin and passaged twice a week. 293 T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, Verviers, Belgium) supplemented with 10% FCS and 10 μg/ml gentamicin. All cells were maintained at 37 °C and 5% $CO_2$.

*Patient*

Patient M17001 started in July 1996 a HAART-regimen containing the NRTIs zidovudine (AZT) and lamivudine (3TC) and the protease inhibitor (PI) Ritonavir (RTV) after irregularly AZT monotherapy. RTV was used irregularly in the first 4 months, and even when RTV treatment was reinitiated the viral load remained above 10,000 copies/ml. After 90 weeks the patient was switched to stavudine (d4T), didanosine (ddI) and Saquinavir (SQV). Two years later the protease inhibitor RTV was reinitiated. Nevertheless, the HIV-RNA plasma concentration remained very high and quite stable. After an initial increase, the CD4 count subsequently decreased over time (Fig. 1).

*RNA isolation and amplification*

HIV-1 nucleic acids were isolated essentially according to the method described by Boom et al. (1990). Briefly, samples were resuspended in lysis buffer containing silica (NucliSens®, Organon Teknika, Boxtel, The Netherlands) and incubated for 10 min at room temperature to let the nucleic acids bind to the silica. The samples were washed twice with washing buffer (NucliSens), twice with ethanol and once with acetone (Merck) before the nucleic acids were eluted in 100 μl poly(A) RNA (40 ng/μl).

The extracted HIV-1 RNA was reverse transcribed and amplified using a one-tube PCR essentially as described before (Nijhuis et al., 1995). To amplify the N-terminal part of RT primers RT18 (5′-GGA AAC CAA AAA TGA TAG GGG AAA TTG GAG G-3′; nucleotides 2377–2407) and RT21 (5′-CTG TAT TTC TGC TAT TAA GTC TTT TGA TGG-3′; nucleotides 3539–3510) were used. cDNA synthesis was performed for 30 min at 42 °C, followed by reverse transcriptase inactivation and DNA denaturation for 5 min at 95 °C. Subsequently 35 cycles of amplification were performed, consisting of a denaturation step for 1 min at 95 °C, an annealing step for 1 min at 55 °C and an extension step for 2 min at 72 °C, followed by an elongation step of 10 min at 72 °C. The amount of amplified product was further amplified in a nested PCR, using primers RT19 (5′-GGA CAT AAA GCT ATA GGT ACA G-3′; nucleotides 2454–2475) and RT20 (5′-CTG CCA GTT CTA GCT CTG CTT C-3′; nucleotides 3462–3441). DNA was denatured for 5 min at 95 °C, followed by 30 cycles of 1-min denaturation at 95 °C, 1-min annealing at 55 °C and 2-min extension at 72 °C. The amplicons were further elongated for 10 min at 72 °C.

PCR-amplified products were purified using the QIAquick PCR purification kit (Qiagen, Leusden, The Netherlands).

*Sequence analysis*

The nested PCR products were genotypically analyzed using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Purified PCR product (3 μl, if necessary diluted) was added to 2 μl 5× BigDye sequencer buffer, 4 μl Ready Reaction premix, 4 μl primer and 7 μl MilliQ. The sequence program consisted of respectively 1 min at 96 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. The N-terminal part of RT was sequenced using primers RT19, RT20, KRT (5′-CAG GAT GGA GTT CAT AAC-3′, nucleotides 3258–3241), BRT (5′-GGA TGG AAA GGA TCA CC-3′, nucleotides 3003–3019) and BRRT (5′-GGT GAT CCT TTC CAT CC-3′, nucleotides 3019–3003). All sequence reactions were analyzed on an ABI Prism® 377 DNA Sequencer and Sequence Navigator 1.0.1 software (Perkin Elmer).

*Clonal sequence analysis*

Nested PCR products generated with primers RT19 and RT20 were ligated in the pGEM-T easy vector (Promega) according to the manufacturer's instructions. *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) were used for transformation. Colonies were picked and cultured overnight at 37 °C in 5 ml LB medium supplemented with ampicillin (40 μg/ml). The plasmid was isolated using the QIAGEN Plasmid Mini Kit (Qiagen) and PCR amplified using the primers RT19 and RT20 as described before. Subsequently, the clones were genotypically analyzed using primers RT19, RT20, BRT, BRRT and KRT as described previously.

*Recombinant virus stocks*

The generation of the recombinant virus stocks was performed essentially as described before (Boucher et al., 1996).

The amount of infectious virus in a viral stock ($TCID_{50}$) in PBMC was determined by the method of Japour et al. (1993) and Reed and Muench (1938).

The 184T reference strain was described by Keulen et al. (1997).

*Site-directed mutant*

Generation of a site-directed mutant was performed using the wild type NRT-vector as described previously (Van Maarseveen et al., 2006). Briefly, recombinant virus was generated by amplification, digestion and ligation of the N-terminal part of RT (amino acids 25–314) in an HXB2 wild type backbone. Primer R70A-RT (5′-TAA TTT TCT CCA TGC ATT ACT-3′, nucleotides 2771–2751) was used together with primers RT-BalI (5′-ATG GCC CAA AAG TTA AAC AAT GG-3′; 2599–2621) and RT-21 to change the second arginine at position 70 into an alanine.

Correct viral clones, determined by sequence analysis, were transfected in 293T cells to obtain the site-directed mutant virus.

*Replication competition experiments*

For determination of the relative replication capacity of the viruses, competition experiments were performed.

Replication competition experiments in PBMC were performed by mixing two recombinant viruses at a ratio of 25:75 and 50:50 based on $TCID_{50}$. In a total volume of 1 ml, $5 \times 10^6$ PHA-stimulated PBMC were infected at a multiplicity of

infection (m.o.i.) of 0.0015. After 2 h of infection at 5% $CO_2$ and 37 °C, cells were washed and subsequently cultured in 10 ml fresh complete culture medium supplemented with 10 U/ml IL-2. The level of virus production was determined using a HIV-1 p24 antigen ELISA as described. If the amount of p24 exceeded 20 ng/ml, 1 ml virus supernatant was used for infection of fresh $5 \times 10^6$ PHA-stimulated PBMC until 5 passages. RNA was extracted from culture supernatant at several time points and the N-terminal part of RT was amplified and sequenced as described before. The amount of mutant compared to the original variant in the population was determined by estimating the relative peak heights of the electropherograms.

Replication competition experiments in Sup-T1 cells were performed essentially as described for PBMC. However, $2 \times 10^6$ Sup-T1 cells were infected at an m.o.i. of 0.001 and virus supernatant was passaged when full-blown syncytia were present in the culture.

### *In vitro evolution experiments*

*In vitro* evolution experiments were performed in PBMC using a recombinant virus clone containing the novel insertion. At an m.o.i. of 0.001, $5 \times 10^6$ PHA-stimulated PBMC were infected with this virus clone for 2 h, subsequently washed and resuspended in 10 ml complete culture medium supplemented with 10 U/ml IL-2. If necessary, the virus culture was replenished with fresh culture medium and/or cells. Virus production was measured by p24 antigen ELISA and 1 ml virus supernatant was used for infection of fresh $5 \times 10^6$ PHA-stimulated PBMC when the amount of p24 was higher than 20 ng/ml. At several time points, RNA was extracted from culture supernatant and the N-terminal part of RT sequenced as described before (Van Maarseveen et al., 2006).

### *Drug susceptibility analysis*

The susceptibility for several RTIs in PBMC was determined by infecting PHA-stimulated PBMC at an m.o.i. of 0.001 in a rotator at 5% $CO_2$ and 37 °C in a total volume of 1 ml. After 2 h, the cells were washed twice and resuspended in complete culture medium supplemented with 10 U/ml IL-2. Serial dilutions of the nucleoside analogue were made in complete culture medium supplemented with IL-2 and added to the infected cells in triploid. After 7-day incubation at 5% $CO_2$ and 37 °C, the viral supernatant was inactivated by Empigen (final concentration of 0.05%; Calbiochem, La Jolla, CA, USA) and incubation for 30 min at 56 °C. The amount of virus production was determined using an HIV-1 p24 antigen ELISA as described before (Moore et al., 1990; McKeating et al., 1991). Briefly, D7320 (Biochrom) was used as coating antibody, BC1071-AP (Aalto) as alkaline-phosphatase-conjugated monoclonal antibody and the AMPAK™ amplification system kit (Dako, Cambridgeshire, UK) was used for detection. Phenotypic resistance was determined by comparing the 50% inhibitory concentration ($IC_{50}$) with a wild type reference strain (HIV-1 HXB2) as described previously (Boucher et al., 1996).

The susceptibility for AZT in MT-2 cells was performed essentially as described by Boucher et al. (1996).

### *Replication capacity under drug pressure*

To determine the replicative fitness of the viruses under drug pressure we have infected $2 \times 10^5$ PBMC at an m.o.i. of 0.001 as described before and incubated the infected cells with a serial dilution of a nucleoside analogue and measured the level of virus production after 7 days by a p24 antigen ELISA as described previously.

### *Modeling of the 5-residue insert*

In order to investigate possible structural effects of the insertion mutant modeling studies were undertaken. The KGSNR insert was introduced into the coordinates of the RT-DNA catalytic complex (Huang et al., 1998) using the molecular graphics program 'O' (Jones et al., 1991). Manual model rebuilding was initially used to remove clashes and then minimization carried out by simulated annealing molecular dynamics in CNS (Brunger et al., 1998).

### *Estimation of in vivo viral fitness*

The establishment of the insertion mutant in the patient was detected by sequencing 3 clones and 2 mixtures from samples 4 and 5 each (week 233 and 263 after start of HAART): none of the sequences contained the insertion mutant in the first sample, and all sequences contained it in the latter. To investigate the fitness advantage of the 5-amino-acid insertion *in vivo*, two samples intermediate of samples 4 and 5; samples 4A and 4B (weeks 236 and 247 after start of therapy), were used for extensive clonal sequence analysis. From both samples 20 viral clones were generated, each containing a unique RT sequence. We obtained a maximum likelihood estimate for the frequency of the mutant at each sampling from the observed composition of the sequenced clones by a Bayesian procedure. The obtained data series were then used to estimate the relative fitness advantage of the insertion mutant by the algorithm developed by Bonhoeffer et al. which considers also the standard deviation of each point and corrects for fluctuations in the overall virus load (Bonhoeffer et al., 2002).

### *Determination of reverse transcriptase activity*

To determine the reverse transcriptase activity of multiple recombinant virus clones, we used the colorimetric reverse transcriptase kit (Roche Applied Science) according to the manufacturer's instruction. To correct for the amount of virus in each stock, 20 ng p24, as determined by a HIV-1 p24 ELISA as previously described, was used as input in the assay.

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